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TITLE: Therapeutic Targeting of TRPV1 for the Treatment of Chronic Pain
Associated with Prostate Cancer Bone Metastasis

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14. ABSTRACT Prostate cancer (PCa) is the most common cause of cancer in men, with advanced form of this cancer frequently leads to bone metastasis, resulting in moderate to severe chronic pain in the back, pelvis and hips, thereby affecting the survivorship and quality of life in these patients. However, the currently available analgesics do not provide effective pain management under these pathological conditions. We proposed to study the precise cellular and molecular mechanisms that underlie nociceptor sensitization and pain sensation associated with bone-metastasized PCa. Specifically, our study is aimed at determining the role of prostate cancer bone metastasis-specific inflammatory factors, IL-6 and TNF- α , PTHrP and ET-1 on upregulation of TRPV1 channel function/expression, and nociceptor sensitization. Further, to test this hypothesis <i>in vivo</i> , we proposed to determine the role of IL-6/TNF- α /PTHrP/ET-1 in mediating pain sensitization in <i>scid</i> mice with xenografts of human PCa cells that metastasize to bones. By far our results show that IL-6/TNF- α /PTHrP/ET-1 sensitize TRPV1 channel activity in sensory neurons that innervate bones. Our results also show that in the presence of IL-6/TNF- α /PTHrP/ET-1, the TRPV1 channel could be activated at mild acidic pH conditions that are hallmarks of metastatic bone tumor microenvironment. Such modulations and mild acid activation of TRPV1 could lead to constitutive sensory neurons firings; thereby provide a mechanism for chronic pain associated with bone-metastasized PCa. Our results on bone-related pain behavior assessments in <i>scid</i> mouse xenografts of human PCa cells, 22Rv1-luc cells, identified several chronic and un-evoked pain behaviors in these mice, specific to bone-metastasized tumor growth.					
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INTRODUCTION:

Prostate cancer (PCa) is the most common cause of cancer in men, with 60-80% subjects susceptible to bone metastasis and subsequent tumor growth of androgen-independent PCa cells. Bone metastasized PCa tumor growth leads to moderate to severe chronic pain in the back, pelvis and hips, due to a combination of tumor destruction of bones and constriction of nearby nerves or the spinal cord, which severely impacts the survivorship and quality of life (QoL) of these patients. Thus, pain relief is a key therapeutic goal to improve the QoL of men suffering from advanced PCa. Unfortunately, the currently available analgesics, such as opioid derivatives, bisphosphonates and non-steroidal anti-inflammatory drugs, do not provide adequate pain relief, due to lack of specificity and dose-limiting side-effects, which even include tumor growth-promoting effects, ultimately resulting in inadequate management of pain in patients with advanced bone-metastasized PCa. The major obstacle in the effective treatment of metastatic bone cancer pain has been our lack of understanding of the precise cellular/molecular mechanisms by which bone-metastasized PCa cells and the surrounding bone marrow microenvironment induce and maintain chronic pain. The overall goal of our study is to determine the precise cellular and molecular mechanisms that underlie nociceptor sensitization and pain sensation associated with bone-metastasized PCa. We are testing the hypothesis that inflammatory mediators and osteolytic/vasoactive peptides, such as interleukin-6 (IL-6), tumor necrosis factor- α (TNF- α), endothelin-1 (ET-1) and parathyroid hormone-related peptide (PTHrP), that are abundant in the metastatic bone tumor microenvironment, sensitize to adjacent sensory nerve fibers by modulation of the transient receptor potential vanilloid-1 (TRPV1) channel on these fibers. Our preliminary study found that sensory neurons that send nerve fibers to bones express the receptors for these mediators/peptides, and sustained activation of these receptors could lead to long-lasting upregulation of TRPV1 expression and function. Such modulations of TRPV1 would lead to constitutive channel activation and sensory neuron firing under patho-physiological conditions, a mechanism that presumably underlies the chronic pain sensation associated with bone metastasized PCa, without any overt stimulation. We proposed to test this hypothesis with two specific aims: 1) Determine the role of prostate cancer bone metastasis-specific inflammatory factors, IL-6 and TNF- α , PTHrP and ET-1 on upregulation of TRPV1 channel function/expression, and nociceptor sensitization. We proposed to employ patch-clamp electrophysiology, ratiometric Ca^{2+} imaging, and biochemical analysis of TRPV1 channel protein in cultured mouse dorsal root ganglia (DRG) neurons, as well as in DRG neurons co-cultured with 22Rv1 cells to address this question. 2) Determine IL-6-, TNF- α -, PTHrP- and ET-1-mediated pain sensitization in *scid* mice with xenografts of human prostate cancer cells that metastasize to bones. We proposed to utilize the human prostate cancer cell line 22Rv1-luc that stably express luciferase, which when grafted into severe combined immune-deficient (*scid*) mice metastasize to and grow tumors in bones with considerably frequency. Alongside monitoring bone metastasis/tumor-growth by bioluminescence imaging, we proposed to assess a battery of bone-related pain behaviors in these *scid* mouse xenografts of 22Rv1-luc cells. Further, we proposed to utilize *in vivo* pharmacological blockade of TRPV1, and IL-6/TNF- α /PTHrP/ET-1 signaling, as well as to utilize 22Rv1-luc xenografts in *scid* mice lacking TRPV1, and subsequent assessments of bone-related pain behaviors to address this question. Our proposed study is aimed at identifying TRPV1 as a therapeutic target for the effective management of chronic pain associated with bone-metastasized PCa. Results from our studies utilizing mouse models of human PCa bone metastasis and chronic pain would serve as a precursor for the follow-up pre-clinical trial of small molecule antagonists of TRPV1 for the treatment of metastatic bone cancer pain. With high incidences of bone-metastasized PCa in men in the US defense personnel, which significantly compromise their job efficiencies and QoL due to chronic pain, results from our study will accelerate the development of effective pain therapeutics for them.

BODY:

Summarized below are the accomplishments from research work performed in the 1st yr of this project.

Milestone-1: Determine the role of prostate cancer bone metastasis-specific inflammatory factors, IL-6/TNF- α /PTHrP/ET-1 on upregulation of TRPV1 channel function/expression, and nociceptor sensitization (Aim 1).

Major Goal/Objective 1: Determine the precise modulation of TRPV1 channel activity in mouse sensory neurons by prostate cancer bone metastasis-specific inflammatory factors, IL-6/TNF- α /PTHrP/ET-1.

Task 1. Acute sensitization of TRPV1 channel activity by IL-6/TNF- α /PTHrP/ET-1 (Aim 1.1; months 1-18). Primary culture of DRG sensory neurons from adult mice, were used in these *in vitro* electrophysiological and Ca²⁺ imaging studies.

1a. Determine IL-6/TNF- α /PTHrP/ET-1-mediated sensitization of TRPV1 currents in mouse sensory neurons in response to activation by capsaicin and acidic pH (months 1-6).

Accomplishments: We have performed these experiments and observed that extracellular perfusion of IL-6 (10 nM), TNF- α (20 nM), PTHrP (10 nM), and ET-1 (100 nM) led to significant potentiation of TRPV1 currents activated by capsaicin (50 nM; Figure 1A,C) and protons (extracellular buffer pH 6.4; Figure 1B,D) in cultured mouse DRG neurons. We also performed similar experiments with the combined application of 1 nM each of IL-6, TNF- α , PTHrP and ET-1 to cultured mouse DRG neurons and observed a ~10-fold increase in pH 6.4-activated TRPV1 currents (Figure 1E). However, extracellular application of IL-6, TNF- α , PTHrP and ET-1 (1 nM each, combined) to cultured DRG neurons from *TRPV1*^{-/-} mice did not lead to any significant change in the fast-activating proton-induced currents (Figure 1E), which are conducted through acid-sensing ion channels. This indicates that IL-6/TNF- α /PTHrP/ET-1-mediated potentiation of mild-to-moderate acidic pH-induced currents in mouse DRG neurons are specific to TRPV1 channel. These results are important in the light of elevated levels of these inflammatory mediators and osteolytic/vasoactive peptides, as well as acidic pH conditions in metastatic PCa bone tumor microenvironment. Currently, we are undertaking experiments to determine the role of such modulations in TRPV1 on sensory neuron firing properties, specifically to mild-to-moderated acidic pH conditions, that are relevant to metastatic PCa bone tumor microenvironment.

1b. Determine the effects of IL-6/TNF- α /PTHrP/ET-1 on the capsaicin and pH dose-dependent activation of TRPV1 currents in mouse sensory neurons (months 4-10).

Accomplishments: We performed these experiments and observed that extracellular perfusion of IL-6, TNF- α , PTHrP, and ET-1 (10 nM for each) led to leftward shifts (2- to 5-fold) in the capsaicin dose-response relationship of TRPV1 currents in cultured mouse DRG neurons. Specifically, the effects of PTHrP and ET-1 on capsaicin dose-dependence of TRPV1 activation were significantly accelerated (~4-fold), as compared to that of IL-6 (~2-fold) and TNF- α (~2.5-fold). Similar observations were also made on pH dose-dependence of TRPV1 activation in cultured mouse DRG neurons upon extracellular perfusion of IL-6, TNF- α , PTHrP, and ET-1 (10 nM for each). Proton-induced TRPV1 currents were absent DRG neurons until the pH reached 6.0 or more acidic; however with the perfusion of these mediators, significant amplitudes of TRPV1 current were recorded even at pH 6.8. These results are particularly important for the pH dose-dependence of TRPV1 activation, since mild-to-moderate acidic conditions (pH 7.0 to 6.5) prevail in the metastatic bone tumor microenvironment. Under physiological conditions, TRPV1 is not activated at this pH range, however, in the presence of elevated levels of IL-6, TNF- α , PTHrP, and ET-1, the channel are now robustly activated in this pH range. This provides one of the key mechanistic bases for constitutive activation of TRPV1 on sensory afferents adjacent to metastatic bone tumor microenvironment.

1c. Determine the effects of IL-6/TNF- α /PTHrP/ET-1 on the temperature activation threshold of TRPV1 currents in mouse sensory neurons (months 8-14).

Accomplishments: Currently we are continuing with these set of experiments are on the halfway through finishing this task. As per our observations so far, a significant decrease in the temperature activation threshold of TRPV1 in mouse DRG neurons is seen upon extracellular perfusion of PTHrP and ET-1 (10 nM for each). Upon completion of all the experiments under this task, data will be analyzed and statistical tests will be performed to determine the significance of these results in the light of constitutive activation of TRPV1 at body

temperatures, under conditions of elevated levels of inflammatory mediators and osteolytic/vasoactive peptides in the metastatic bone tumor microenvironment. Detailed results of these sets of experiments will be provided in the next annual report, along with the data on temperature-induced sensory neuron firings.

- 1d. Determine the effects of IL-6/TNF- α /PTHrP/ET-1 on TRPV1 channel-mediated Ca^{2+} influx in mouse sensory neurons by ratiometric functional Ca^{2+} imaging (months 12-18).

Accomplishments: Although, these sets of experiments were proposed to be undertaken during the months of 12 to 18, we have already accomplished this task by now. We performed functional Ca^{2+} imaging experiments on cultured mouse DRG neurons with two consecutive application of capsaicin (50 nM) with or without the intermittent perfusion of IL-6 (10 nM), TNF- α (20 nM), PTHrP (10 nM), and ET-1 (100 nM), which led to significant potentiation of capsaicin-induced Ca^{2+} influx (Figure 2A-B). These results constitute yet another conformation of the sensitizing effects of these bone metastasis specific elevated levels of inflammatory mediators and osteolytic/vasoactive peptides.

Milestone-2: Determine the role of upregulation of TRPV1 expression/activity mediated by prostate cancer bone metastasis-specific inflammatory factors, IL-6/TNF- α /PTHrP/ET-1, on development/sensitization of chronic bone-related pain, utilizing *scid* mouse xenografts of human prostate cancer cells that show bone metastasis; as well as pharmacological and genetic validation of TRPV1 as the key target for alleviating pain in the metastatic prostate/bone cancer-bearing mice (Aim 2).

Major Goal/Objective 1: Development of *scid* mouse xenografts of human prostate cancer cells, 22Rv1, and characterization/assessment of bone-related chronic pain behavior in these mice.

Task 1. Assessment of bone-related pain behaviors in *scid* mice with 22Rv1 xenografts (Aim 2.1; months 4-12). Cultures of human prostate cancer cell line, 22Rv1-luc (stably expressing luciferase) and human prostate epithelial cell line, RWPE-1-luc (stably expressing luciferase), were used in these *in vivo* xenograft/behavior studies.

- 1a. Generation of *scid* mice xenografts of saline-injected or uninjected, as well as of 22Rv1-luc and RWPE-1-luc cells, weekly monitoring of tumor metastasis/growth by bioluminescence imaging (BLI), and weekly bone-related pain behavior assessments (months 4-7).

Accomplishments: We utilized *Rag1-scid* mice (C57BL/6 background) for the generation of human PCa xenografts with metastatic tumor growth in long bones. Our first set of experiments consisted of 10 animals each for uninjected, saline-injected, 22Rv1-luc, and RWPE-1-luc (all intracardiac injections). Out of the ten 22Rv1-luc-injected mice only 2 developed metastatic tumor growth in unilateral hind limbs, 5 developed tumors in the thoracic region, 2 developed tumors in the adrenals, and 1 did not develop any tumor. Using BLI, metastasis and tumor growth was first detected on the 3rd week after cell injection, and BLI signal intensities showed progressive increase in subsequent weeks, until the week 7. Uninjected, saline-injected and RWPE-1-luc-injected mice did not show any BLI signal starting from week 0 to week 7 after cell injection. We performed bone-related pain behavioral assessments (as detailed in the original research proposal) on these 40 mice, once before the cell injections, and thereafter once a week after the cell injection, upto the 7th week. The person performing behavioral assessments was blinded to individual animal groups and status of tumor growth. The second cohort of xenografts, with similar group distribution, is currently in the mid-way of the experiment.

- 1b. Histopathological and radiological analyses of tumor growth and bone destruction, as well as analysis of TRPV1 expression in DRG in *scid* mice xenografts mentioned above in the sub-task (1a) (months 7-9).

Accomplishments: After the behavioral assessments of bone-related pain in *Rag1-scid* mice xenografts on the 7th week post cell injection, we euthanized these animals and performed *ex vivo* analysis of tumor growth using BLI, followed by radiological analysis of bone destruction. So far two of the *scid* mice xenografts of 22Rv1-luc human PCa cells that exhibited

metastatic tumor growth in hind limb bones showed considerable bone destruction, although no fractures were observed. Further, we performed whole animal perfusion with fixative, and subsequently removed the femur and tibia-fibula for tissue sectioning and H&E, as well as immunostaining for nerve fiber sprouting. Additionally, we removed both the pairs of L4-L6 DRGs (ipsi- and contra-lateral to metastatic tumor growth) from tumor-bearing and non-tumor mice for subsequent sectioning and immunostaining. Currently, both these tissue section stainings are underway, which we plan to complete in the next three months. In the mean time we have now standardized the procedure for the immunostaining of sensory nerve fibers and protein expression there in by immunohistological stainings of mouse limb bone sections, which we published in the Journal of Visualized Experiments.¹

1c. Thorough analyses (including statistical) of metastatic bone tumor-induced chronic pain behavior in these mouse xenografts (months 10-12).

Accomplishments: Our first cohort of *Rag1-scld* mice xenografts led to only 2 out of 10 mice that showed metastatic tumor growth in hind limb bones. We have performed all the proposed bone-related pain behavior assessments in these, as well as all other mice included in this cohort. The uninjected, saline-injected and RWPE-1-luc injected mice did not show any significant changes in compression-induced hyperalgesia, thermal & mechanical hyperalgesia, thermal & mechanical allodynia, gait parameters, relative weight-bearing on hind paws, and hind limb grip-strengths on the weeks 1, 2, 3, 4, 5, 6, and 7 post cell/saline injection, as compared to their respective baseline values obtained prior to cell/saline injection. In contrast, we observed a visible and progressive decreases in the compression force, thermal & mechanical paw withdrawal latencies/thresholds, and hind limb grip strengths in both the mice with metastatic 22Rv1-luc tumor growth in the limb, starting from week 4 till 7 post cell/saline injections. Additionally, we observed decreased hind limb weight bearing and alterations in several gait parameters (decreased stance duration, decreased break & propulsion durations, increased swing duration, and decreased paw pressure), starting from week 4 till 7 post cell/saline injections. All these changes were observed for the hind limbs ipsilateral to metastatic bone tumor growth. However, due to the fact that the number of animals here with bone metastasis is just 2, there is no scope for a valid statistical comparison of the data here. As mentioned above, the second cohort of xenografts, with similar group distribution, is currently in the mid-way of the experiment, after the completion of which we will compile all the bone-related pain behavioral assessment data for statistical comparison.

KEY RESEARCH ACCOMPLISHMENTS:

- In our first year of this study we found that, inflammatory mediators such as IL-6 and TNF- α , the osteolytic peptide PTHrP, and the vasoactive peptide ET-1, all of which are specifically elevated in the tumor microenvironment on bone-metastasized PCa, specifically upregulate currents through TRPV1 channel in mouse DRG sensory neurons. These neurons send sensory nerve fibers to limb bones, including bone marrow, and are the detectors/mediators of the sensation of chronic pain. The results on PTHrP are of particular interest, since PTHrP has been linked to the establishment of bone metastasis and subsequent tumor growth in PCa. To this end, our study found that modulation of proton-activated currents in mouse DRG neurons in response to the mediators are specific to TRPV1 channel, since no modulations in proton-activated currents were observed in the DRG neurons from *TRPV1*^{-/-} mice.
- Our study found that, in the presence of these mediators the TRPV1 channel on sensory neurons could be activated at mild acidic pH conditions, which prevails in the bone tumor microenvironment. These results are important in the light of constitutive TRPV1 channel activation on sensory nerves adjacent to metastatic PCa bone tumor microenvironment.
- Our study further found that elevated levels of these bone-metastasis specific inflammatory mediators and peptides induce increased Ca²⁺ flux into DRG neurons via the TRPV1 channel. Elevated levels of Ca²⁺ could serve as a precursor for increase in the protein expression of

TRPV1 and other pain-sensing channels/receptors, which will be next tested during the course of this study.

- Our preliminary assessment of bone-related pain behaviors in *scid* mouse xenografts of 22Rv1-luc human PCa cells suggest progressive changes in relative weight-bearing, grip-strength, gait parameters and thermal & mechanical hypersensitivities, from the 3rd week onwards post tumor cell injection. These changes were observed only ipsilateral to tumor-bearing hind limbs, and are absent in control, saline-injected, and non-malignant human prostate epithelial cells, RWPE-1-luc-injected animals, as well as in *scid* mice injected with 22Rv1-luc cells showing tumors growing in the thoracic, adrenal, and mandible regions.

REPORTABLE OUTCOMES:

Manuscripts:

Shepherd A.J., and **Mohapatra D.P.** (2012). Tissue Preparation and immunostaining of mouse sensory nerve fibers innervating skin and limb bones. *Journal of Visualized Experiments*. Vol. 59: e3485.¹

This publication was an outcome of our thorough standardization of the procedure for immunostaining of sensory nerve fibers and protein expression there in by immunohistological stainings of mouse limb bone sections, as well as of mouse skin sections.

Scientific Presentations:

I (the PI) chaired and moderated a scientific symposium entitled “Should we mine new pain-transducing ion channels or focus on specific properties of known ion channel targets?” at the 31st Annual Meeting of the American Pain Society, held at Honolulu, HI, May 16-19, 2012. In this symposium, I presented our data on specific functional modulations in TRPV1 by a wide variety of inflammatory cytokines, chemokines and bioactive peptides that are elevated in the metastatic PCa bone tumor microenvironment, and the mechanistic bases of chronic pain associated with metastatic bone cancers.

CONCLUSION:

In conclusion, our results from the 1st year of this study have led us to determine that inflammatory mediators and osteolytic/vasoactive peptides, the levels of which are specifically elevated in the metastatic PCa bone tumor microenvironment, sensitize the TRPV1 channel in sensory neurons that innervate the bones. Our study also determined that in the presence of IL-6, TNF- α , PTHrP and ET-1 the TRPV1 channel could be activated at mild acidic pH conditions that are characteristic features of metastatic bone tumor microenvironment. Such modulations and mild acid activation of TRPV1 could lead to constitutive sensory neurons firings; thereby provide a mechanism for chronic pain associated with bone-metastasized PCa. Our results on bone-related pain behavior assessments in *scid* mouse xenografts of human PCa cells, 22Rv1-luc cells, identified several chronic and un-evoked pain behaviors in these mice, specific to bone-metastasized tumor growth. Currently, we are continuing with our studies to determine the mechanistic bases for metastatic PCa bone tumor-induced upregulation of TRPV1 expression and sensory neuron firing, as well as determining the role of TRPV1 in chronic bone-related pain behaviors in *scid* mouse xenografts of 22Rv1-luc cells.

REFERENCES:

1. Shepherd A.J., and **Mohapatra D.P.** (2012). Tissue Preparation and immunostaining of mouse sensory nerve fibers innervating skin and limb bones. *J Vis Exp*. Vol. 59: e3485.



Video Article

Tissue Preparation and Immunostaining of Mouse Sensory Nerve Fibers Innervating Skin and Limb Bones

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Abstract

Detection and primary processing of physical, chemical and thermal sensory stimuli by peripheral sensory nerve fibers is key to sensory perception in animals and humans. These peripheral sensory nerve fibers express a plethora of receptors and ion channel proteins which detect and initiate specific sensory stimuli. Methods are available to characterize the electrical properties of peripheral sensory nerve fibers innervating the skin, which can also be utilized to identify the functional expression of specific ion channel proteins in these fibers. However, similar electrophysiological methods are not available (and are also difficult to develop) for the detection of the functional expression of receptors and ion channel proteins in peripheral sensory nerve fibers innervating other visceral organs, including the most challenging tissues such as bone. Moreover, such electrophysiological methods cannot be utilized to determine the expression of non-excitatory proteins in peripheral sensory nerve fibers. Therefore, immunostaining of peripheral/visceral tissue samples for sensory nerve fibers provides the best possible way to determine the expression of specific proteins of interest in these nerve fibers. So far, most of the protein expression studies in sensory neurons have utilized immunostaining procedures in sensory ganglia, where the information is limited to the expression of specific proteins in the cell body of specific types or subsets of sensory neurons. Here we report detailed methods/protocols for the preparation of peripheral/visceral tissue samples for immunostaining of peripheral sensory nerve fibers. We specifically detail methods for the preparation of skin or plantar punch biopsy and bone (femur) sections from mice for immunostaining of peripheral sensory nerve fibers. These methods are not only key to the qualitative determination of protein expression in peripheral sensory neurons, but also provide a quantitative assay method for determining changes in protein expression levels in specific types or subsets of sensory fibers, as well as for determining the morphological and/or anatomical changes in the number and density of sensory fibers during various pathological states. Further, these methods are not confined to the staining of only sensory nerve fibers, but can also be used for staining any types of nerve fibers in the skin, bones and other visceral tissue.

Video Link

The video component of this article can be found at <http://www.jove.com/video/3485/>

Protocol

1. Animal Perfusion

All animal procedures performed in this study are approved by the Institutional Animal Care and Use Committee of the University of Iowa, and follow NIH guidelines for the use of animals in research.

1. On the day before perfusion, prepare 1 L of phosphate buffer (0.2 M PB in double distilled H₂O, pH 7.4), and store at 4°C. This will be used for perfusion and post-fixation processes.
2. On the day of perfusion, prepare 500 ml of 4.0% paraformaldehyde in 0.1M PB (PFA, pH 7.4) fixative solution, a volume sufficient for the perfusion of 2 mice: microwave 200 ml of ddH₂O in a glass beaker for 30 sec or until it approaches boiling. Add 20 g of granular paraformaldehyde (PFA) to the beaker with constant stirring under a fume hood. Add 5 ml of 5 N NaOH drop-wise and stir until the solution clears. When the PFA is completely dissolved, cool the solution to room temperature (RT). Slowly add 250 ml of 0.2 M PB while stirring continuously. Using progressively weaker HCl solutions (6 N to 1 N to 0.1 N), adjust the pH to 7.4. Adjust the final volume to 500 ml and chill on ice. Also prepare 400 ml of 0.1 M PB from 0.2 M stock by diluting 1:1 in ddH₂O and chill on ice.
3. Anesthetize the mouse by intraperitoneal injection of an overdose of anesthetic (sodium pentobarbital, 80 mg/kg, administered with a 27G needle).
4. During perfusion, 50 ml of 0.1 M PB (dilute 0.2 M PB stock 1:1 with ddH₂O) and 150 ml of PFA will be passed sequentially into the mouse circulation. Set up the peristaltic pump by filling the tubing with PB and fixing a 23^{1/2}G butterfly needle at one end. Immerse the other end of the tubing into the beaker containing 0.1 M PB. Set the speed of the peristaltic pump to 10 ml/min, and pump through a sufficient amount of the solution to ensure that there are no air bubbles in the tubing.

5. Wait until the anesthetized mouse no longer shows any reflex activity, such as toe/tail pinch and blink reflex. Lay the animal on a dissection tray ventral side up inside a fume hood and secure the paws with tape. Spray the fur with 70% ethanol. Make an incision through the skin along the midline to expose the ribcage and the uppermost quarter of the abdominal wall. Then, cut through the abdominal wall at the base of the ribcage. After making this incision, the diaphragm and lower end of the sternum should be visible. Carefully cut from left to right through the margin of the diaphragm, and along the full length of the sternum, taking care not to damage the lungs. Some minor bleeding from the sternum at this stage is normal and will not compromise perfusion. Once the incision along the sternum has been made and the diaphragm cut, it should be possible to lift each half of the ribcage upwards and outwards, such that the heart is exposed. Move aside the lungs if necessary. Both halves of the ribcage can be held in this position with the use of hemostatic clamps. Insert the butterfly needle (23^{1/2}G) attached to the peristaltic pump 3-4 mm into the left ventricle, parallel to the long axis of the animal. This placement minimizes the risk of the needle becoming dislodged. Make a small incision in the right atrium to allow the return circulation to flow out of the heart.
6. Begin perfusion with 0.1 M PB at 10 ml/min for 4-5 min. If there is still a significant amount of blood emanating from the right atrium at this point, continue until the flow is clear.
7. Stop the flow of PB and switch the inlet on the peristaltic pump to the 4% PFA solution. Reduce the flow rate to 5 ml/min and resume pumping PFA solution for 20-25 min. As the PFA enters the circulation, the muscles go into spasm and, after a few minutes, the animal should be literally "fixed" in position. This rigidity can be tested by gently pushing against the hindpaws, taking care not to move the animal and dislodge the cannula. Good perfusion will prevent the limb from flexing in response. Once perfusion with PFA is complete, the cannula and hemostatic clamps can be disconnected and the cadaver prepared for tissue dissection. The dissection approach used depends on the specific tissue.
8. Prepare 4% PFA / 5% (v/v) picric acid (PA; for collection and post-fixation of plantar punch only) by adding saturated picric acid solution to 4% PFA. Inclusion of PA significantly improves antigen detectability in peripheral neuronal tissues¹.
9. Prepare bone/cartilage tissue decalcifying/cryoprotectant solution - 10% EDTA in 0.1 M PB with 0.07% glycerol and 15% sucrose. EDTA-based solutions decalcify tissue whilst preserving epitopes².
10. Prepare the cryoprotectant solution - 30% sucrose in 0.1 M PB.

2. Tissue Dissection/Removal, Post-fixation and Sectioning

1. Plantar Punch

Place the perfused animal ventral side down on a cutting mat or other sturdy surface. Holding the foot plantar surface-up, press down firmly with the punch biopsy tool (3 mm diameter; Harris micro-punch, Ted Pella Inc.) into the middle of the foot. Turn the biopsy tool back and forth through 180 degrees to confirm the biopsy tool has cut through the entirety of the hind paw. Gently remove the biopsy tool from the foot and eject the tissue into sterile 2 ml tube with cap, containing 1 ml of 4% PFA / 5% PA solution.

2. Limb Bone (ex. Femur)

Make a lateral incision along the back of the animal at the level of the pelvis, continuing down along both hind limbs. Cut into the pelvis and surrounding muscle to separate the femur from the pelvis whilst leaving the proximal head of the femur intact. Cut into the tibia/fibula to leave the distal head intact, removing the surrounding muscle/periosteum from the bone shaft. Place the femur into a 2 ml tube containing 1 ml of 4% PFA / 5% PA solution.

3. Post-fix the tissue samples in 4% PFA / 5% PA solution, with gentle mixing on a rocker for 16-18 h at 4°C.
4. Decalcify the tissue as follows: *For plantar punch* (to decalcify the small bones of the foot) place the tissue punch in a sterile 2 ml tube with cap, containing 1.5 ml of 10% EDTA solution in 0.1 M PB with 0.07% glycerol and 15% sucrose. Place the tube on a rocker with mild mixing for 16-18 h at 4°C. *For limb bones* place the tissue in a sterile 2 ml tube with cap, containing 1.5 ml of 10% EDTA solution in 0.1 M PB with 0.07% glycerol and 15% sucrose. Place the tube on a rocker with mild mixing for 6-7 days at 4°C. The decalcification solution should be changed every 24 hours and the tissue monitored for loss of rigidity with a pair of forceps.
5. Transfer the tissue into a sterile 2 ml tube with cap with 1.5 ml of cryoprotectant solution (30% sucrose in 0.1 M PB), and place the tube on a rocker with mild mixing for 16-18 h at 4°C.
6. Prepare tissue for sectioning: place a bed volume of optimal cutting temperature (OCT) compound (Sakura Finetek USA Inc.) on a cryostat tissue mounting block and allow to freeze in the cryostat chamber (typically maintained at -20°C). A cryogenic aerosol (Cyto-freeze, Control Co. USA) can also be sprayed on the OCT to accelerate the freezing process. Place tissue specimen on this bed of OCT and cover with an additional thin layer of OCT. Gently spray this OCT with cryogenic aerosol until it has hardened and the tissue within has frozen. Place specimen onto the cutting head in the cryostat chamber and allow the tissue specimen block to equilibrate to the cutting temperature - at least 1 hour. Trying to section when the embedding compound is still too cold results in brittle sections and tissue damage.
7. Once the tissue has reached optimal cutting temperature, begin cutting the specimen and generate 40 µm sections.
8. *For plantar punch* collect the cryosections into 12-well tissue culture plates containing 0.1 M PB with 10 mM sodium azide. Make sure that the sections remain submerged in this solution at 4°C. Sections can be stored in this manner for up to 4-6 months for immunostaining purposes. Alternatively, free-floating sections can be stored indefinitely in cryoprotectant solution at -20°C (500 ml 0.1 M PBS, pH 7.2, 30 g sucrose, 10 g PVP40, 300 ml ethylene glycol). Adjust final volume to 1L with distilled water³. *For limb bones* collect the cryosections directly onto gelatin pre-coated slides (or Superfrost Plus slides, Fisher Scientific) and allow to air-dry for 1 h, before storing at -20°C. Bone sections on slides stored in this way can be used for immunostaining purposes within 2-3 months.

3. Immunostaining of Tissue Sections for Sensory Nerve Fibers

3.1. Plantar punch sections - floating section staining

1. Using a razor blade, cut 6-7 mm from the end of a 1 ml micropipette tip. Pre-condition the inside of the tip by aspirating 1% fetal bovine serum (FBS) in 0.1 M PB several times (this inhibits sticking of the tissue sections to the walls of the tip). Transfer plantar punch sections to be stained to a 24-well tissue culture plate. Normally 8-10 sections should be stained per well to ensure several high-quality sections are generated per immunostaining.
2. Wash plantar punch sections with 500 µl of 0.1 M PB per well for 5 min with vigorous mixing on a rocker at RT. Repeat 2 more times.

3. Discard the washing solution and incubate the plantar punch sections in blocking solution, consisting of 10% goat serum and 0.3% Triton X-100 in 0.1 M PB (500 μ l of blocking solution per well), with gentle mixing on a rocker for 1 h at 4°C.
4. Discard the blocking solution and incubate the tissue sections in primary antibody/antibodies diluted in 250 μ l blocking solution for 18-24 h with gentle mixing on a rocker at 4°C. Based on investigator's experimental requirements, single immunolabeling (with one primary antibody against a specific protein or nerve fiber marker), or double immunolabeling (with two primary antibodies against two proteins or nerve fiber markers) can be performed on the same section. While performing double immunolabeling it is important to verify that the two primary antibodies used are raised in different host species (e.g. one raised in mouse and one raised in rabbit), or alternatively, purified monoclonal antibodies of different immunoglobulin G (IgG) isotypes (e.g. one IgG1 and one IgG2a) can be used. It is advisable to seal the plate with Parafilm for overnight incubations to avoid excessive evaporation. In order to stain the peripheral sensory nerve fibers, several specific antibodies can be used. Antibodies against the protein neurofilament 200 (NF200; Sigma) label large-diameter fibers, whereas calcitonin gene-related peptide (CGRP; Sigma) and transient receptor potential vanilloid 1 (TRPV1, Neuromics) label peptidergic, small-diameter peripheral sensory fibers. All peripheral nerve fibers can also be stained with antibodies against β 3-tubulin. In skin, collagen IV (Abcam) is used to delineate the basement membrane that separates the epidermis from the dermis. As a general rule, antibodies need to be 5-10 times more concentrated than for cultured cell-based immunofluorescence assays, typically at a working concentration of 5-10 μ g/ml.
5. Wash the tissue sections with 500 μ l of blocking solution (with Triton X-100) per well for 5 min with vigorous mixing on a rocker at RT. Repeat 3 more times.
6. Discard the washing solution and incubate tissue sections in appropriate fluorophore-conjugated secondary antibodies (typically diluted 1:1000 in blocking solution, 500 μ l) with gentle mixing on a rocker at 4°C. The plate must be wrapped in aluminium foil to avoid photobleaching of fluorophores.
7. Wash the tissue sections with 500 μ l of blocking solution per well for 10 min with vigorous mixing on a rocker at RT. Then wash with 500 μ l of 0.1 M PB with vigorous mixing for 10 min at RT. Finally, wash with 500 μ l of 0.05 M PB with vigorous mixing for 10 min at RT.
8. Using a 1 ml micropipette with an FBS-treated tip (cut 6-7 mm from the end), aspirate the sections from the tissue culture plate and transfer onto SuperFrost Plus microscope slides. Arrange the sections and absorb excess wash buffer with a fine paintbrush. Avoid prolonged exposure to light. Incubate the slides at RT, in order to allow sections to dry sections on slide (5-30 min).
9. Apply several drops of mounting medium (ProLongGold, Vectashield or similar), slowly place a glass coverslip onto sections. Allow the slides to sit in darkness for 5 min, and then seal the coverslip edges with transparent nail polish. Allow air-drying for 15-20 min. The slides can now be visualized or stored at -20°C for several years without any significant loss of fluorescence.

3.2. Limb bone sections - on-slide staining

1. Allow the slides to return to RT from -20°C storage. Using a hydrophobic barrier pen (Super Pap Liquid Blocking Pen, Ted Pella Inc.) circumscribe the region on the slide containing the bone sections to be stained with a generous layer of solution. Allow to air dry for 10-15 min.
2. Wash the bone sections with 250 μ l of 0.1 M PB per slide for 5 min and repeat for 2 more times.
3. Discard the washing solution and incubate the bone sections in blocking solution, consisting of 10% goat serum and 0.3% Triton X-100 in 0.1 M PB (250 μ l of blocking solution per slide) for 1 h at 4°C.
4. Discard the blocking solution and incubate the bone sections in primary antibody (or combination of primary antibodies, in case of double immunolabeling as mentioned under 3.1.4) diluted in 250 μ l blocking solution for 18-24 h at 4°C. It is very important to note that the slides must be placed in a humidified chamber with a sealed lid, in order to avoid drying of primary antibody solution. In order to stain the peripheral sensory nerve fibers, the above-mentioned set of antibodies (see section 3.1.4) can be used with similar working concentrations.
5. Wash the tissue sections with 250 μ l of blocking solution (with Triton X-100) per slide for 15 min at RT and repeat for 3 more times.
6. Discard the washing solution and incubate bone sections in appropriate fluorophore-conjugated secondary antibodies (typically diluted 1:1000 in blocking solution, 250 μ l) at 4°C. The staining chamber must be wrapped in aluminium foil to avoid photobleaching of fluorophores. For convenience, it is advisable to use dark-colored staining chambers (e.g. Slide incubation tray/box, RPI Corp.).
7. Wash the bone sections with 250 μ l of blocking solution per slide for 15 min at RT. Then wash with 500 μ l of 0.1 M PB for 15 min at RT. Finally, wash with 500 μ l of 0.05 M PB for 15 min at RT. Dip briefly in ddH₂O to rinse slides and then incubate at RT in order to allow the bone sections to air dry (5-30 min). Avoid prolonged exposure to light.
8. Apply several drops of mounting medium (ProLongGold or similar) and slowly place a glass coverslip onto sections. Let the slides stand in darkness for 5 min, and then seal the coverslip edges with transparent nail polish. Allow drying for 15-20 min. Slides can be stored at -20°C for several years without loss of fluorescence.

4. Representative Results

4.1. Plantar punch sections

Plantar punch tissue sections can be visualized under epifluorescence microscope, or under a confocal microscope with a 10X, 40X or 63X objective.

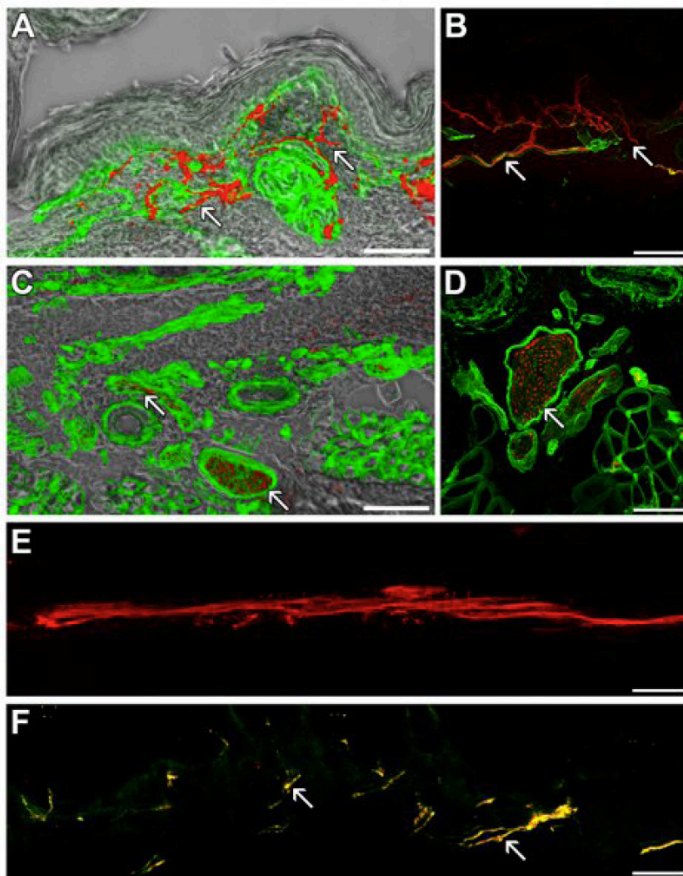


Figure 1. A-D show staining with Collagen IV antibody (green) in basement membranes, at the epidermal-dermal junction and in cartilage and muscle. Numerous CGRP- (A-B, red) and NF200-positive fibers (C-D, red) are distributed throughout the mouse skin in the plantar region (arrows). β 3-tubulin is a pan-neuronal marker (E, red), whereas TRPV1 staining (F; red) is mainly confined to small-diameter fibers that are also CGRP-positive (green; arrowheads). A and C are epifluorescence images taken with a 10X objective (scale bar -500 μ m); B, D, E and F are confocal image composites generated from an 11-image z-stack taken at 2 μ m increments under a 60X objective (scale bar - 50 μ m).

4.2. Limb bone sections

Limb bone tissue sections can also be visualized under epifluorescence microscope, or under a confocal microscope with a 10X, 40X or 63X objective.

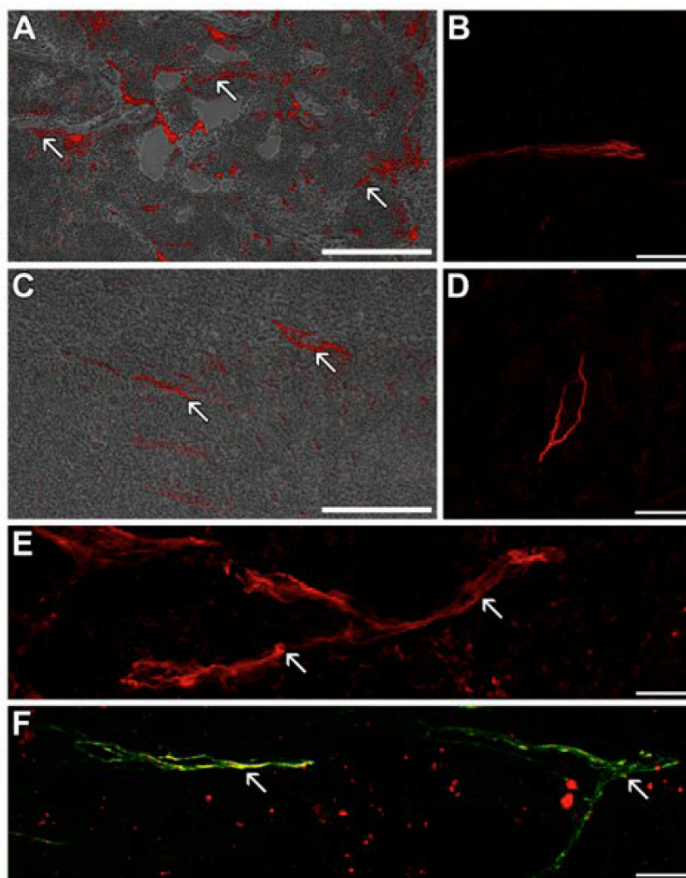


Figure 2. shows immunostaining with anti-CGRP (A-B, red; arrows), anti-NF200 (C-D, red; arrows), anti- β 3-tubulin (E; red) and anti-TRPV1 (F; red) co-stained with anti-CGRP antibody (green; arrows). These images show subtypes of sensory nerve fibers distributed throughout the bone matrix in the spongy head region of mouse femur. A and C are epifluorescence images taken with a 10X objective (scale bar - 500 μ m); B, D, E and F are confocal image composites generated from an 11-image z-stack taken at 2 μ m increments under a 60X objective (scale bar - 50 μ m).

Discussion

Here we have detailed the methods for preparation of mouse skin and bone tissue sections for immunostaining and detection of peripheral sensory nerve fibers. The sections produced from plantar punch biopsies contain both glabrous and hairy skin, which means the protocol can be used on any skin type. These techniques can also be employed to stain other cell types in these tissues (e.g. leukocytes, vascular endothelia, smooth muscle among others). These methods provide an excellent compromise between optimal ultrastructural preservation (which is achieved by glutaraldehyde fixation, but frequently results in disruption of epitopes and diminished immunostaining staining quality) and immunocytochemical detectability, if the procedures are followed step-by-step in a rigorous manner.

Detection of sensory nerve fibers in these tissues can aid in our understanding of the regulation of peripheral neurite outgrowth and sprouting⁴, as well as anatomical changes in peripheral sensory afferents under different pathological conditions. Furthermore, changes in the expression of neurotransmitters, receptors, ion channels or other phenotypic markers in normal developmental or pathological conditions can also be studied^{3,5-10}. Along with appropriate electrophysiological, biochemical and behavioral testing, such changes in peripheral sensory neuron staining patterns can be used to test hypotheses related to various pain states^{6,9}, inflammation¹¹ and neuropathies^{5,12}. In conclusion, these techniques provide an invaluable source of *in vivo* data that complements and reinforces other anatomical, structural and functional data acquired through additional approaches, furthering our understanding of the regulation and acquisition of plasticity in peripheral sensory nerve fibers in health and disease.

Disclosures

No conflicts of interests declared.

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SUPPORTING DATA:

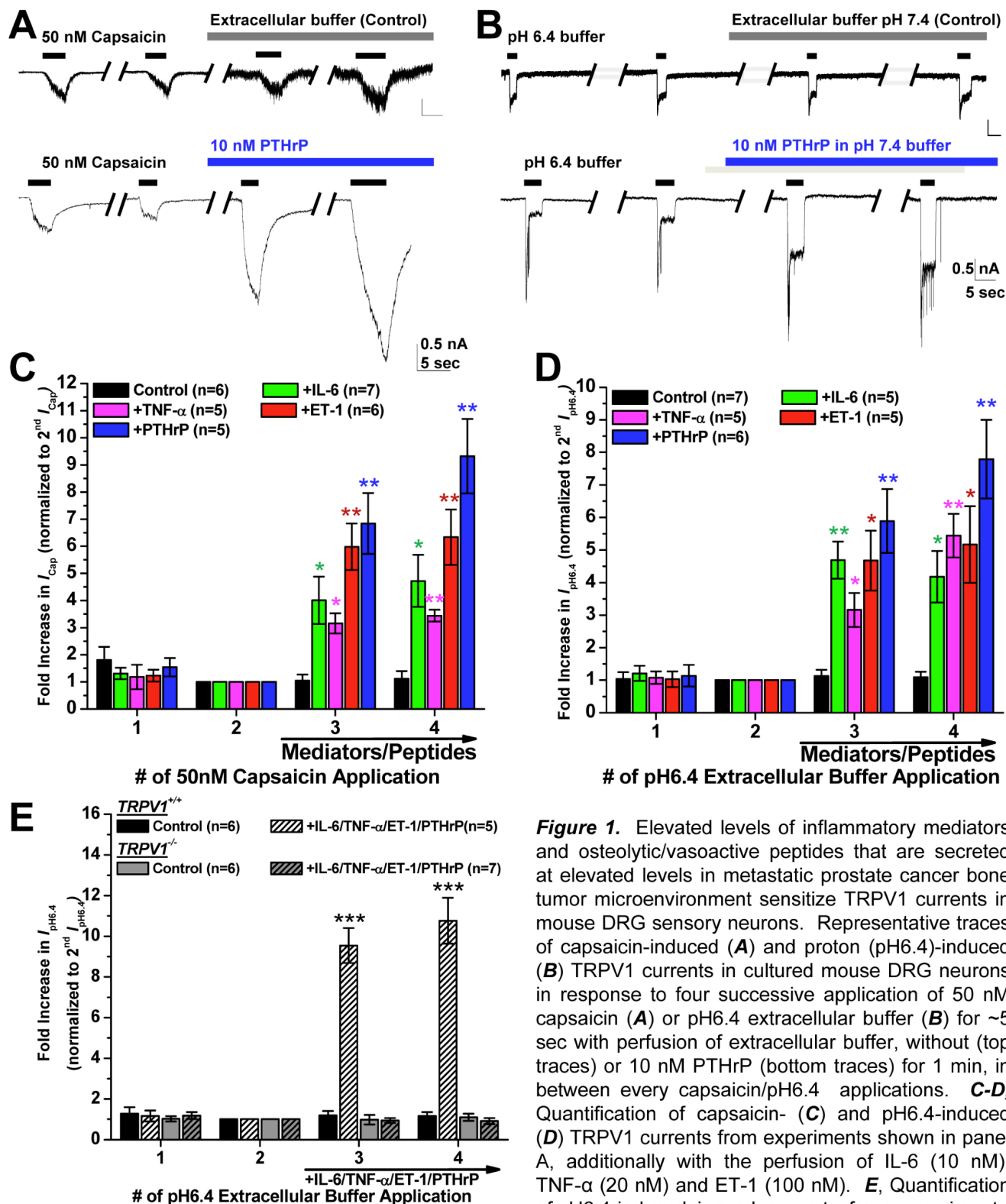


Figure 1. Elevated levels of inflammatory mediators and osteolytic/vasoactive peptides that are secreted at elevated levels in metastatic prostate cancer bone tumor microenvironment sensitize TRPV1 currents in mouse DRG sensory neurons. Representative traces of capsaicin-induced (**A**) and proton (pH6.4)-induced (**B**) TRPV1 currents in cultured mouse DRG neurons in response to four successive application of 50 nM capsaicin (**A**) or pH6.4 extracellular buffer (**B**) for ~5 sec with perfusion of extracellular buffer, without (top traces) or 10 nM PTHrP (bottom traces) for 1 min, in between every capsaicin/pH6.4 applications. **C-D**, Quantification of capsaicin- (**C**) and pH6.4-induced (**D**) TRPV1 currents from experiments shown in panel A, additionally with the perfusion of IL-6 (10 nM), TNF- α (20 nM) and ET-1 (100 nM). **E**, Quantification of pH6.4-induced inward currents from experiments

on cultured DRG neurons from *TRPV1*^{+/+} and *TRPV1*^{-/-} mice, with the perfusion of IL-6, TNF- α , ET-1, and PTHrP (1 nM combined). Data are presented as mean \pm SEM of peak currents at each agonist episodes, normalized to the 2nd peak current episodes. The “n” numbers are shown in parentheses for each treatment group. * p <0.05, ** p <0.01 and *** p <0.001, significantly different compared to their respective control group mean currents in that particular episode of agonist application (One way ANOVA with post-hoc Dunnett’s correction).

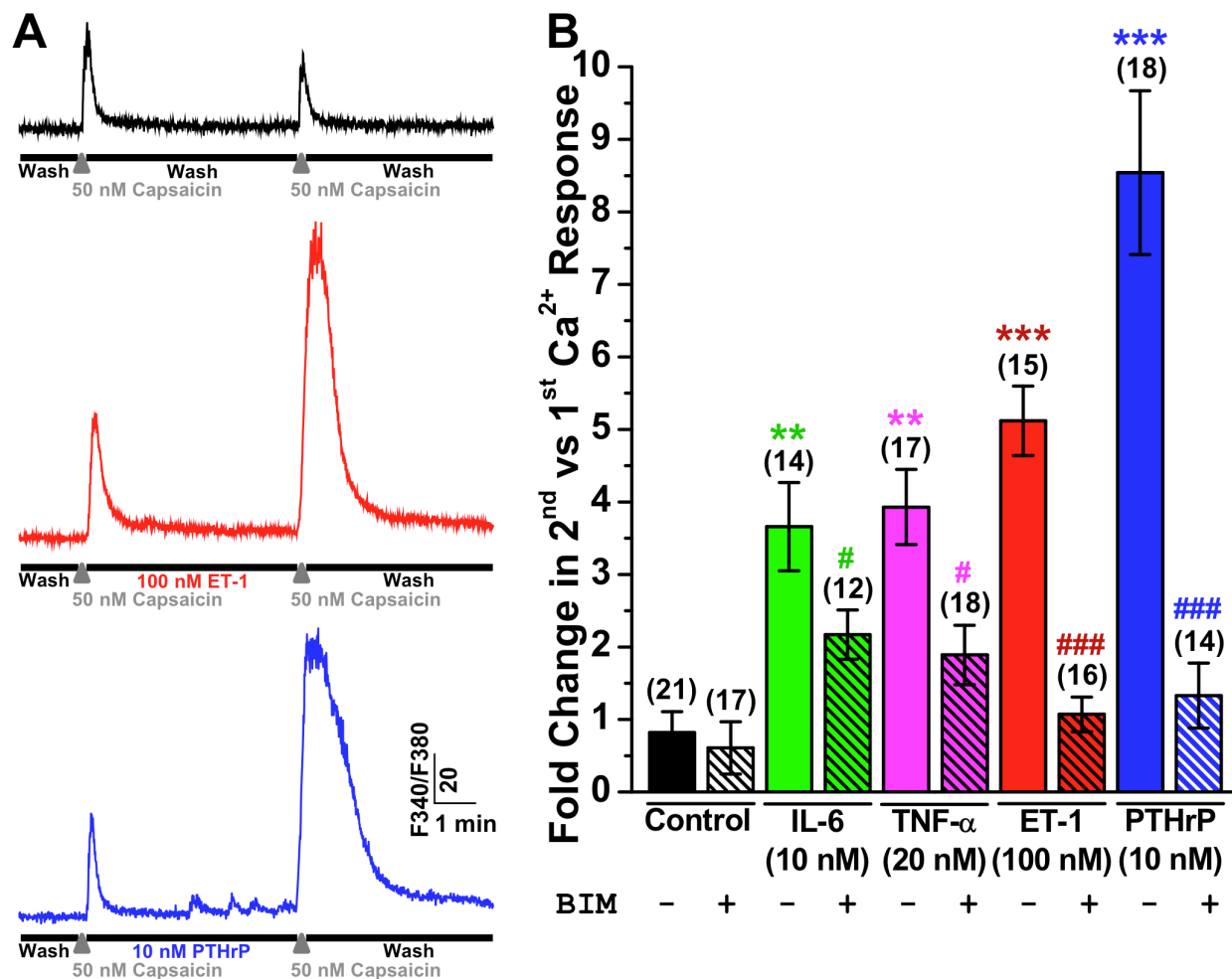


Figure 2. Elevated levels of inflammatory mediators and osteolytic/vasoactive peptides that are secreted at elevated levels in metastatic prostate cancer bone tumor microenvironment sensitize TRPV1-mediated Ca^{2+} influx into mouse DRG sensory neurons. **A**, Representative traces of capsaicin-induced Ca^{2+} influx in cultured mouse DRG neurons in response to two successive application of 50 nM capsaicin (15 sec) with perfusion of extracellular buffer, without (top trace) or with 100 nM ET-1 (middle trace), and 10 nM PTHrP (bottom trace) for 5 min, in between the two capsaicin applications. Cells were loaded with Fura-2-AM Ca^{2+} indicator dye, then excited alternately at 340 and 380 nm, and emitted fluorescence were collected at 510 nm. **B**, Quantification of capsaicin-induced Ca^{2+} flux measurements from the experiments shown in panel A, additionally with the perfusion of IL-6 (10 nM) and TNF- α (20 nM). In another set of experiment, these mediators were applied on neurons pretreated and co-treated with the PKC inhibitor bis-indoylmelamide-I (BIM-I; 500 nM). Data are presented as mean \pm SEM of the ratio of 2nd vs 1st Ca^{2+} peak F_{340}/F_{380} signal. The “n” numbers are shown in parentheses for each treatment group. ** p <0.01 and *** p <0.001, significantly different compared to control group solid bars; and # p <0.05 and ### p <0.001, significantly different compared to their respective solid bars (One way ANOVA with post-hoc Dunnett’s correction).